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PRIMARY STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTOR

ANNUAL REPORT

JAMES W. PATRICK

AUGUST 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5198

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution limited to US Government Agencies only; (~~Pricetary~~ Proprietary Information, Contractor Performance Evaluation, December 2, 1987).
DECLASSIFICATION/DOWNGRADING SCHEDULE	
PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
NAME OF PERFORMING ORGANIZATION The Salk Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
ADDRESS (City, State, and ZIP Code) P.O. Box 85800 San Diego, California 92138	7b. ADDRESS (City, State, and ZIP Code)
NAME OF FUNDING/SPONSORING ORGANIZATION US Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable) SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5198
ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M1 61102BS11	AA	068

TITLE (Include Security Classification)
Primary Structure of Nicotinic Acetylcholine Receptor

PERSONAL AUTHOR(S)
James W. Patrick

TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 7/15/85 TO 7/14/86	1986 August	37

SUPPLEMENTARY NOTATION

COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	13		
06	01		

ABSTRACT (Continue on reverse if necessary and identify by block number)

Cholinergic transmission occurs between nerve and muscle and between nerve and nerve. In both cases, acetylcholine is released by the nerve, bound by a nicotinic acetylcholine receptor, and destroyed by the enzyme acetylcholinesterase. Our goal has been to isolate cDNA clones encoding the nicotinic acetylcholine receptor at the neuromuscular junction and to use these clones to determine the structure of the receptor. In this report we present the amino acid sequence of the muscle nicotinic acetylcholine receptor and show that the clones we have isolated will direct the synthesis of the receptor. We have also used the clones to isolate related clones, which we argue encode the receptors found at synapses in the central nervous system. For one of these clones, we have determined its sequence and the distribution in the brain of the transcript.

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DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☑ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code) 301-663-7325 / 22c. OFFICE SYMBOL SGRD-RMI-S

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SUMMARY

This study had two specific goals. The first was to obtain the primary structure for the subunits of a mammalian nicotinic acetylcholine receptor and the second was to isolate and sequence cDNA clones encoding the nicotinic acetylcholine receptors found in the central nervous system. Both of these goals have been realized. We have isolated cDNA clones encoding the α-, β-, γ- and δ-subunits of the mouse muscle nicotinic acetylcholine receptor and have determined the sequences for the α-, β-, and γ-subunits. The sequence of the δ-subunit was completed in another laboratory. The clones were engineered to make them suitable for expression studies and inserted into plasmids downstream of a promotor. These plasmids have been used to synthesize RNA which will direct the synthesis of functional acetylcholine receptors in the *Xenopus* oocyte.

We used our clones encoding the muscle acetylcholine receptor α-subunit to screen by low stringency DNA/DNA hybridization a cDNA library prepared from a neuronal cell line. We isolated three clones out of one million examined and determined the sequence of the one with the longest insert. This sequence revealed that the clone encoded a protein with considerable structural homology to the muscle receptor α-subunits. We have been able to demonstrate by *in situ* hybridization that transcripts hybridizing to the clone are expressed in regions of the brain known to have cholinergic transmission. Based upon this evidence, we have proposed that the clone encodes the α-subunit of a neuronal nicotinic receptor.

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BACKGROUND

A major goal in the neurosciences is to understand how the communication between cells in the central nervous system determines behavior. To a large extent, we understand the general principles of the communication process. We know that neurotransmitters are released by the presynaptic cell and that receptors on the postsynaptic cell bind the neurotransmitters and generate an electrical event in the postsynaptic cell. We know that some of these electrical events are inhibitory and some are excitatory, and we know ionic mechanisms that are capable of generating both kinds of responses. We have also identified several different neurotransmitters and neurotransmitter receptors, but we know little about how this diversity might produce different responses to a single kind of neurotransmitter.

Our understanding of neurotransmission in the central nervous system is in part limited by the complexity of the system. The cells are small and may have thousands of synapses derived from neurons from many different parts of the brain. However, the abundance of synapses does not mean that the receptors are present in large quantities. In fact, the actual quantity is low and purification of receptors from the brain is difficult at best. Consequently, neither cellular nor biochemical approaches to studying neurotransmission within the brain is particularly promising. This is unfortunate because our best handle on the function of the brain may well be through modification of neurotransmission. Drugs that affect the activation of neurotransmitter receptors are potent modulators of behavior. However, testing drugs in the intact animal is difficult because so little is known about the various sites of actions of the test compounds. Ideally, one would identify all of the neurotransmitter receptors of a given type and determine their distribution throughout the known functional domains of the brain. One might then engineer the expression of these receptors in cell lines where their function could be assayed accurately and individually. One could then detect differential drug sensitivities and design drugs that differentially modulate receptor activity in specific functional regions of the brain.

As described in the present report, we have isolated cDNA clones encoding the four subunits of the mouse muscle nicotinic acetylcholine receptor and have engineered these clones to permit their expression in the *Xenopus* oocyte. In this system, we have explored the functional requirements for the individual subunits and have begun experiments to modify receptor function by mutation. These results demonstrate the feasibility of studying the properties of nicotinic acetylcholine receptors using RNA transcribed from cDNA clones and provide a foundation both for future studies of these receptors. They also establish the criteria to be applied in the analysis of neuronal nicotinic receptors. Clearly the ultimate criteria for a receptor is function and there are now established methodologies available for studying function of proteins encoded by recombinant DNA clones.

We have also used our clones encoding the muscle receptor to isolate clones encoding receptors from the central nervous system. We have found and sequenced two clones encoding α-subunits and have shown that the genes encoding these proteins are expressed in different parts of the rodent brain. It is not yet clear that these clones encode subunits of neuronal nicotinic acetylcholine receptors because we have not yet been able to apply the criteria of function discussed above. Clearly, this is an important next step. These results that we have obtained do, however, indicate that we have identified a family of genes encoding nicotinic acetylcholine receptors, that these genes are differentially expressed in the brain, and that we will be able to study the function and pharmacological properties of the gene products in a system free of the complexity of the central nervous system.

I. MOUSE MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR

We began our studies of the molecular biology of the nicotinic acetylcholine receptor using the electric organ of the ray *Torpedo* from which we could obtain large quantities of starting material (for reviews of receptor, see Popot and Changeux, 1984; Stroud and Finer-Moore, 1985). This work led to the isolation of cDNA clones encoding the α- and γ-subunits of the *Torpedo* receptor, determination

of their sequences, and construction of detailed models for the structure of the receptor molecule (Claudio et al, 1983; Patrick et al, 1983). This approach was limited in that the majority of the biophysical and developmental studies had been done in mammalian systems.

We decided to focus on the mammalian system and began by isolating a cDNA clone encoding the α-subunit of the mouse muscle nicotinic acetylcholine receptor (Boulter et al, 1985). The sequence of the α-subunit which we deduced from this clone led to new testable models of the ligand binding domain of the α-subunit. To test these models, we needed clones encoding all four subunits of the muscle acetylcholine receptor in a form suitable for expression in the *Xenopus* oocyte system. The isolation and characterization of the γ-subunit encoding clone were accomplished (Boulter et al, 1986). Dr. Norman Davidson's laboratory isolated a δ-subunit encoding clone which lacked the 5 prime untranslated sequences (LaPolla et al, 1985). We used this clone to isolate from our library a clone suitable for expression. In this report, we document the isolation and sequence of the β-subunit encoding cDNA clone and show the expression of all four clones in the *Xenopus* oocyte. We also report the isolation and sequence of two new clones which we propose encode the α-subunits of nicotinic acetylcholine receptors which mediate synaptic transmission between neurons in the peripheral and central nervous systems.

We screened a mouse muscle λgt10 cDNA library with a probe derived from a cDNA clone coding for the β-subunit of *Torpedo* acetylcholine receptor and isolated two clones which were found to encode the mouse muscle β-subunit. Figure 1 illustrates a partial restriction map and the sequencing strategy for clone BMB265. This clone lacks the initiator methionine and a portion of the leader sequence so a new library was constructed and screened to find clones suitable for expression. Clone BMB49 was isolated from this screening. The partial restriction map and sequencing strategy for this clone are also found in Figure 1.

The results in Figure 2 show the nucleotide and deduced amino acid sequence for the mouse muscle acetylcholine receptor β-subunit derived from clone BMB49. The total length of the cDNA is 2100 nucleotides. Alignment of translations from all

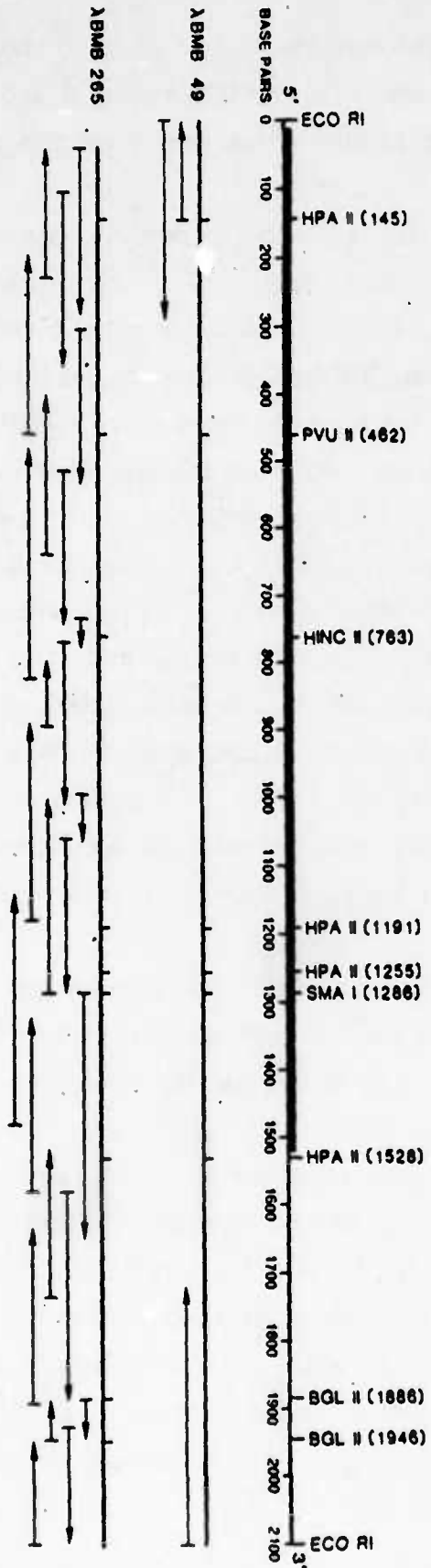
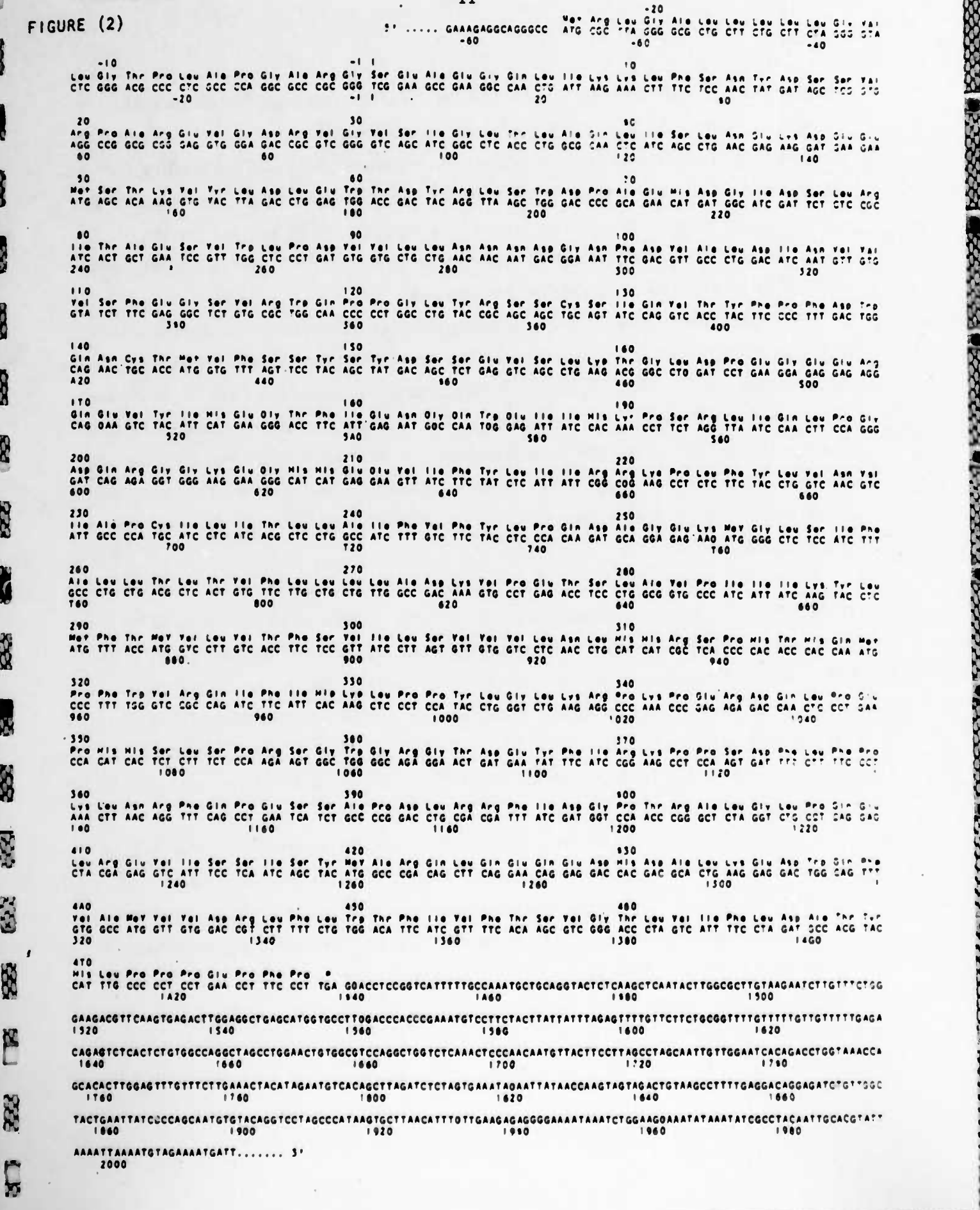

λBMB 265
λBMB 49
BASE PAIRS
5'
0
ECO RI
100
HPA II (145)
200
300
400
PVU II (462)
500
600
700
HINC II (763)
800
900
1000
1100
1200
HPA II (1191)
HPA II (1255)
SMA I (1286)
1300
1400
1500
HPA II (1528)
1600
1700
1800
BGL II (1886)
1900
BGL II (1946)
2000
2100
ECO RI
3'

FIGURE (2)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-60 -60 -40
-10 -1 1 10
Leu Gly Thr Pro Leu Ala Pro Gly Ala Arg Gly Ser Glu Ala Glu Gly Gln Leu Ile Lys Lys Leu Phe Ser Asn Tyr Asp Ser Ser Val
CTC GGG ACG CCC CTC GCC CCA GGC GCC CGC GGG TCG GAA GCC GAA GGC CAA CTG ATT AAG AAA CTT TTC TCC AAC TAT GAT AGC TCG GTG
-20 -1 1 20 30
20 30 40
Arg Pro Ala Arg Glu Val Gly Asp Arg Val Gly Val Ser Ile Gly Leu Thr Leu Ala Gln Leu Ile Ser Leu Asn Glu Lys Asp Glu Glu
AGG CCG GCG CGG GAG GTG GGA GAC CGC GTC GGG GTC AGC ATC GGC CTC ACC CTG GCG CAA CTC ATC AGC CTG AAC GAG AAG GAT GAA GAA
60 80 100 120 140
50 60 70
Met Ser Thr Lys Val Tyr Leu Asp Leu Glu Trp Thr Asp Tyr Arg Leu Ser Trp Asp Pro Ala Glu His Asp Gly Ile Asp Ser Leu Arg
ATG AGC ACA AAG GTG TAC TTA GAC CTG GAG TGG ACC GAC TAC AGG TTA AGC TGG GAC CCC GCA GAA CAT GAT GGC ATC GAT TCT CTC CGC
160 180 200 220
80 90 100
Ile Thr Ala Glu Ser Val Trp Leu Pro Asp Val Val Leu Leu Asn Asn Asn Asp Gly Asn Phe Asp Val Ala Leu Asp Ile Asn Val Val
ATC ACT GCT GAA TCC GTT TGG CTC CCT GAT GTG GTG CTG CTG AAC AAC AAT GAC GGA AAT TTC GAC GTT GCC CTG GAC ATC AAT GTT GTG
240 260 280 300 320
110 120 130
Val Ser Phe Glu Gly Ser Val Arg Trp Gln Pro Pro Gly Leu Tyr Arg Ser Ser Cys Ser Ile Gln Val Thr Tyr Phe Pro Phe Asp Trp
GTA TCT TTC GAG GGC TCT GTG CGC TGG CAA CCC CCT GGC CTG TAC CGC AGC AGC TGC AGT ATC CAG GTC ACC TAC TTC CCC TTT GAC TGG
340 360 380 400
140 150 160
Gln Asn Cys Thr Met Val Phe Ser Ser Tyr Ser Tyr Asp Ser Ser Glu Val Ser Leu Lys Thr Gly Leu Asp Pro Glu Gly Glu Glu Arg
CAG AAC TGC ACC ATG GTG TTT AGT TCC TAC AGC TAT GAC AGC TCT GAG GTC AGC CTG AAG ACG GGC CTG GAT CCT GAA GGA GAG GAG AGG
420 440 460 480 500
170 180 190
Gln Glu Val Tyr Ile His Glu Gly Thr Phe Ile Glu Asn Gly Gln Trp Glu Ile Ile His Lys Pro Ser Arg Leu Ile Gln Leu Pro Gly
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520 540 560 580 600
200 210 220
Asp Gln Arg Gly Gly Lys Glu Gly His His Glu Glu Val Ile Phe Tyr Leu Ile Ile Arg Arg Lys Pro Leu Phe Tyr Leu Val Asn Val
GAT CAG AGA GGT GGG AAG GAA GGG CAT CAT GAG GAA GTT ATC TTC TAT CTC ATT ATT CGG CGG AAG CCT CTC TTC TAC CTG GTC AAC GTC
600 620 640 660 680
230 240 250
Ile Ala Pro Cys Ile Leu Ile Thr Leu Leu Ala Ile Phe Val Phe Tyr Leu Pro Gln Asp Ala Gly Glu Lys Met Gly Leu Ser Ile Phe
ATT GCC CCA TGC ATC CTC ATC ACG CTC CTG GCC ATC TTT GTC TTC TAC CTC CCA CAA GAT GCA GGA GAG AAG ATG GGG CTC TCC ATC TTT
700 720 740 760
260 270 280
Ala Leu Leu Thr Leu Thr Val Phe Leu Leu Leu Leu Ala Asp Lys Val Pro Glu Thr Ser Leu Ala Val Pro Ile Ile Ile Lys Tyr Leu
GCC CTG CTG ACG CTC ACT GTG TTC TTG CTG CTG TTG GCC GAC AAA GTG CCT GAG ACC TCC CTG GCG GTG CCC ATC ATT ATC AAG TAC CTC
780 800 820 840 860
290 300 310
Met Phe Thr Met Val Leu Val Thr Phe Ser Val Ile Leu Ser Val Val Val Leu Asn Leu His His Arg Ser Pro His Thr His Gln Met
ATG TTT ACC ATG GTC CTT GTC ACC TTC TCC GTT ATC CTT AGT GTT GTG GTC CTC AAC CTG CAT CAT CGC TCA CCC CAC ACC CAC CAA ATG
880 900 920 940
320 330 340
Pro Phe Trp Val Arg Gln Ile Phe Ile His Lys Leu Pro Pro Tyr Leu Gly Leu Lys Arg Pro Lys Pro Glu Arg Asp Gln Leu Pro Glu
CCC TTT TGG GTC CGC CAG ATC TTC ATT CAC AAG CTC CCT CCA TAC CTG GGT CTG AAG AGG CCC AAA CCC GAG AGA GAC CAA CTC CCT GAA
960 980 1000 1020 1040
350 360 370
Pro His His Ser Leu Ser Pro Arg Ser Gly Trp Gly Arg Gly Thr Asp Glu Tyr Phe Ile Arg Lys Pro Pro Ser Asp Phe Leu Phe Pro
CCA CAT CAC TCT CTT TCT CCA AGA AGT GGC TGG GGC AGA GGA ACT GAT GAA TAT TTC ATC CGG AAG CCT CCA AGT GAT TTC CTT TTC CCT
1060 1080 1100 1120
380 390 400
Lys Leu Asn Arg Phe Gln Pro Glu Ser Ser Ala Pro Asp Leu Arg Arg Phe Ile Asp Gly Pro Thr Arg Ala Leu Gly Leu Pro Gln Glu
AAA CTT AAC AGG TTT CAG CCT GAA TCA TCT GCC CCG GAC CTG CGA CGA TTT ATC GAT GGT CCA ACC CGG GCT CTA GGT CTG CCT CAG GAG
1080 1160 1180 1200 1220
410 420 430
Leu Arg Glu Val Ile Ser Ser Ile Ser Tyr Met Val Ala Arg Gln Leu Gln Glu Gln Glu Asp His Asp Ala Leu Lys Glu Asp Trp Gln Phe
CTA CGA GAG GTC ATT TCC TCA ATC AGC TAC ATG GTC GCC CGA CAG CTT CAG GAA CAG GAG GAC CAC GAC GCA CTG AAG GAG GAC TGG CAG TTT
1240 1260 1280 1300
440 450 460
Val Ala Met Val Val Asp Arg Leu Phe Leu Trp Thr Phe Ile Val Phe Thr Ser Val Gly Thr Leu Val Ile Phe Leu Asp Ala Thr Tyr
GTG GCC ATG GTT GTG GAC CGT CTT TTT CTG TGG ACA TTC ATC GTT TTC ACA AGC GTC GGG ACC CTA GTC ATT TTC CTA GAT GCC ACG TAC
320 1340 1360 1380 1400
470
His Leu Pro Pro Pro Glu Pro Phe Pro •
CAT TTG CCC CCT CCT GAA CCT TTC CCT TGA GGACCTCCGGTCATTTTTGCCAAATGCTGCAGGTACTCTCAAGCTCAATACTTGGCGCTTGTAAGAATCTTGTTTCTGG
1420 1440 1460 1480 1500
GAAGACGTTCAAGTGAGACTTGGAGGCTGAGCATGGTGCCTTGGACCCACCCGAAATGTCCTTCTACTTATTATTTAGAGTTTTGTTCTTCTGCGGTTTTGTTTTTGTTGTTTTTGAGA
1520 1540 1560 1580 1600 1620
CAGAGTCTCACTCTGTGGCCAGGCTAGCCTGGAACTGTGGCGTCCAGGCTGGTCTCAAACTCCCAACAATGTTACTTCCTTAGCCTAGCAATTGTTGGAATCACAGACCTGGTAAACCA
1640 1660 1680 1700 1720 1740
GCACACTTGGAGTTTGTTTCTTGAAACTACATAGAATGTCACAGCTTAGATCTCTAGTGAAATAGAATTATAACCAAGTAGTAGACTGTAAGCCTTTTGAGGACAGGAGATCTGTTGGC
1760 1780 1800 1820 1840 1860
TACTGAATTATCCCCAGCAATGTGTACAGGTCCTAGCCCATAAGTGCTTAACATTTGTTGAAGAGAGGGGAAAATAAATCTGGAAGGAAATATAAATATCGCCTACAATTGCACGTATT
1880 1900 1920 1940 1960 1980
AAAATTAAAATGTAGAAAATGATT..... 3'
2000

reading frames with calf and *Torpedo* acetylcholine receptor β-subunits shows extensive amino acid homology for an open reading frame of 1506 nucleotides commencing at nucleotide -69 and ending with nucleotide 1437. This reading frame assignment predicts a primary structure for the mouse acetylcholine receptor β-subunit which consists of a 23-amino acid leader peptide and a mature protein of 478 amino acids. Clone BMB49 has 15 nucleotides preceding the initiator methionine and 578 nucleotides of 3' untranslated sequences.

We now have cDNA clones encoding all four subunits of the mouse muscle nicotinic acetylcholine receptor. The amino acid sequences of the four subunits of this receptor are shown in Figure 3. In this figure, the sequences have been aligned to maximize homologies. This emphasizes the conservation of proposed structural domains for the receptor. For example, the proposed membrane-spanning regions are quite conserved between the various subunits. In contrast, the proposed amphipathic helix is less conserved although the amphipathicity of the structure is still evident.

Several of the cDNA clones as originally isolated had extraneous sequences at their 5' ends. These sequences were presumably derived from errors made by the reverse transcriptase during the creation of the library. Since these sequences might have caused initiation of protein synthesis out of the proper reading frame, we engineered each clone to create a suitable 5' sequence and placed the clone downstream of the SP6 promotor. The arrangement of the clones thus created is found in Figure 4. These plasmids are now suitable for synthesis of RNA for injection into the *Xenopus* oocyte.

We synthesized RNA using the clones found in Figure 4 and injected the capped RNA into *Xenopus* oocytes using the procedures first described by Gurden et al., 1971. We assayed synthesis and assembly of a nicotinic acetylcholine receptor .electrophysiologically, by binding α-bungarotoxin, and by immunoprecipitation of the receptor peptides. The results in Figure 5 show a typical voltage trace recorded from an oocyte injected with RNA specific for each of the four subunits. The response is blocked by d-tubocurarine and is dependent upon the concentration of acetylcholine up to the reversal potential. In the future, we will be able to study the

Mouse Nicotinic Acetylcholine Receptor

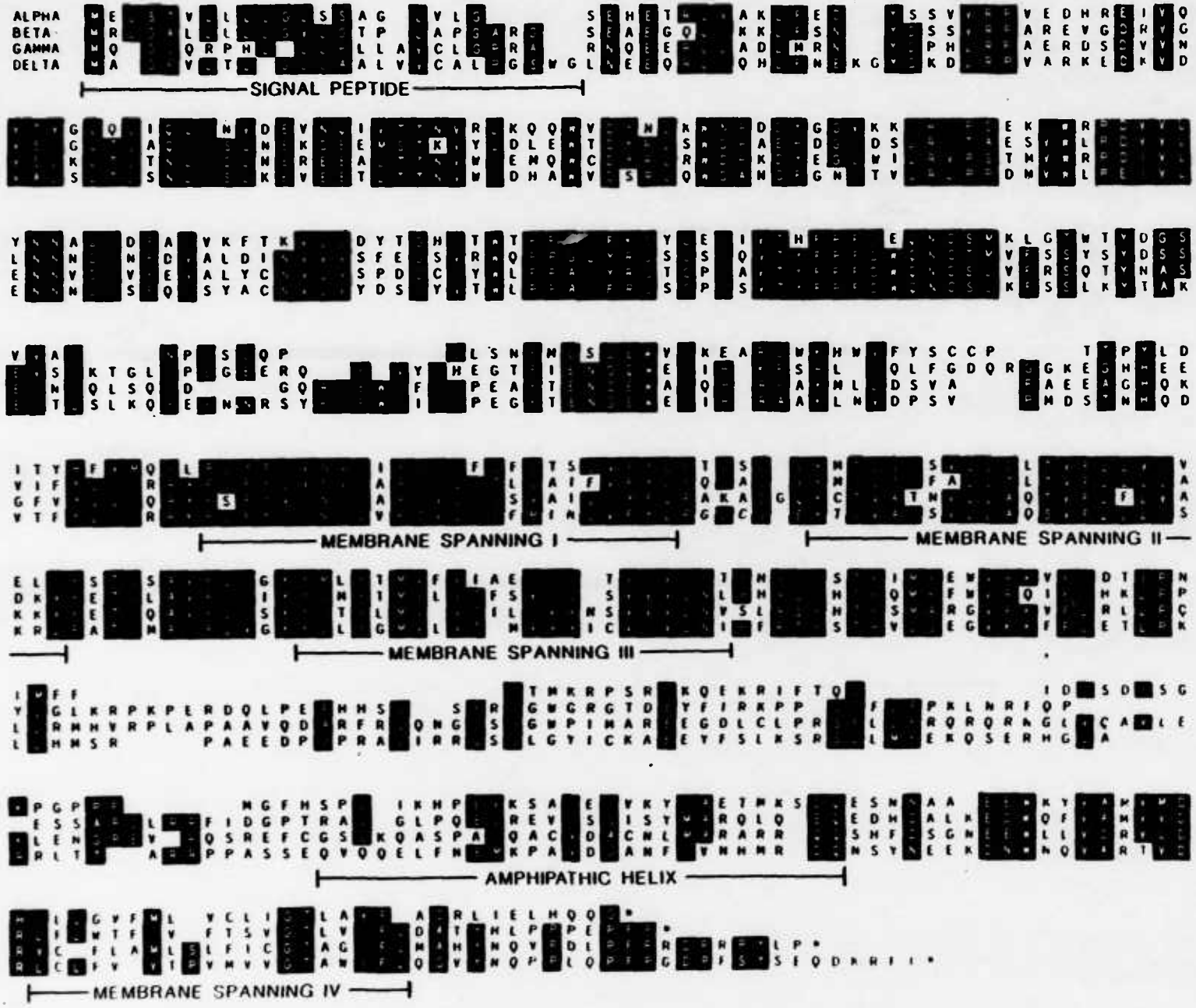

FIGURE (4)

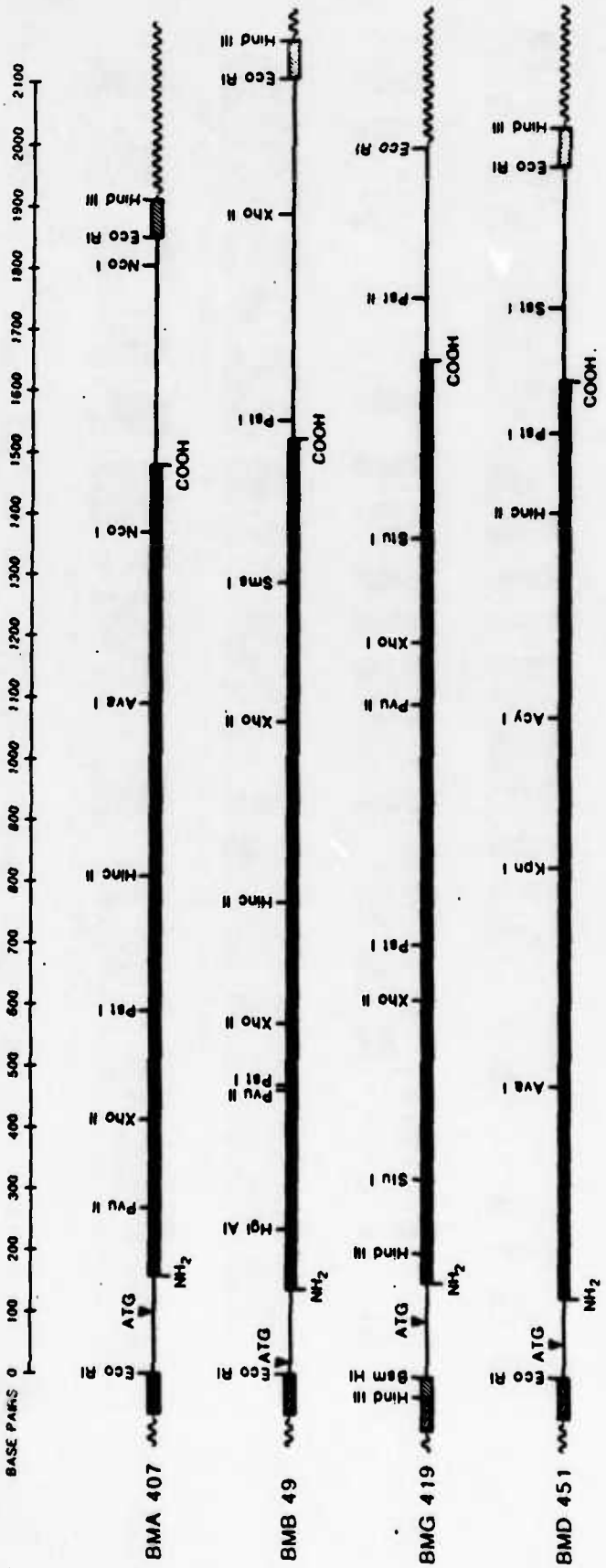

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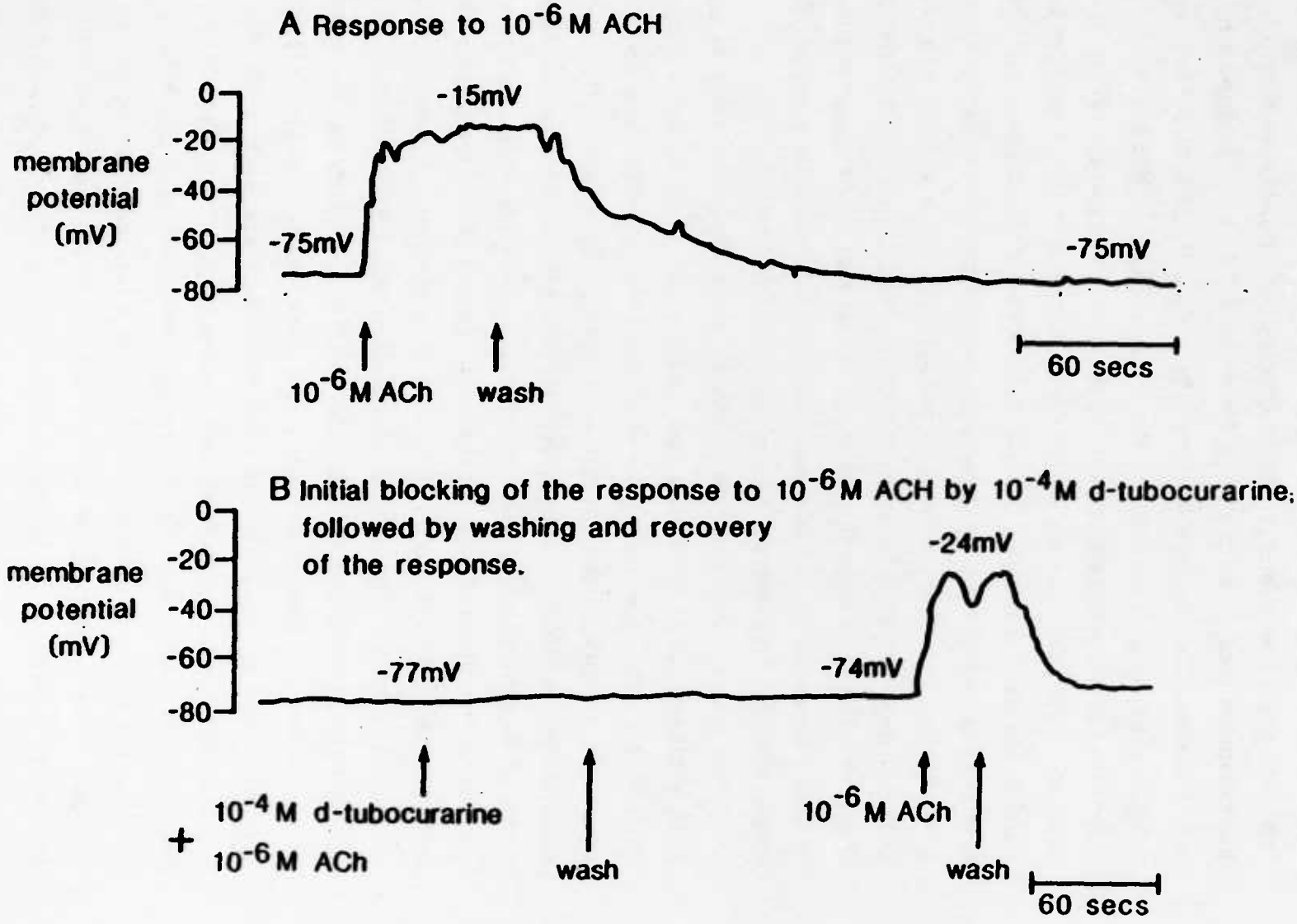
FIGURE (5)

Voltage Responses of Oocyte Injected with mRNA for Mouse α-, β-, γ-, δ-, Subunits to Applied Agonists and Antagonists

(Control Solution: 115mM NaCl, 1.8mM $CaCl_2$, 2.5mM KCl, 10mM Hepes pH 7.2 and 1 µM Atropine).

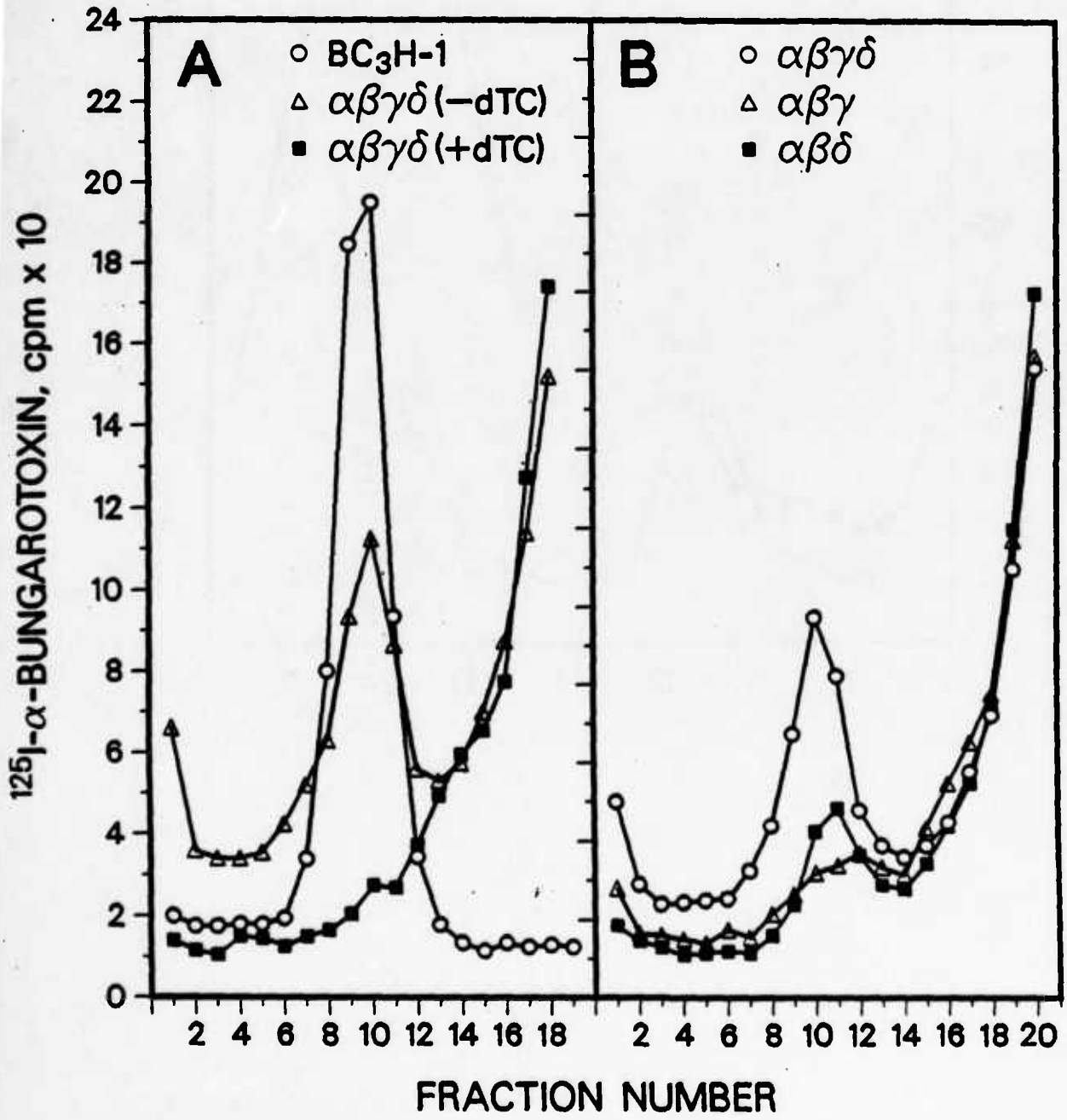


861065

dose-response relationship under voltage-clamp conditions. The results in Figure 6A show sucrose gradient profiles of α-bungarotoxin-labeled detergent extracts of injected eggs. The α-bungarotoxin-binding component sediments with the α-bungarotoxin complexes prepared from the BC_3H-1 cell line used to prepare the cDNA clones. This figure also shows that only a fraction of the total toxin-binding activity is on the cell surface. In the BC_3H-1 cell line, the internal toxin-binding activity is a precursor to the cell surface receptor (Patrick et al., 1977a). It seems likely that this is also the case for the *Xenopus* oocyte, but it has not yet been shown. Finally, we have incubated the injected oocytes in ^{35}S-methionine and prepared detergent extracts from the labeled eggs. The extracts were sedimented on sucrose gradients and pooled fractions were precipitated with anti-receptor antibody. The peak of immunoprecipitable radioactivity sediments as the native receptor (Figure 7). Based on these three assays, we feel that we have successfully produced, in the *Xenopus* oocyte, a functional mouse muscle nicotinic acetylcholine receptor whose synthesis was directed by our subunit cDNA clones.

We are now in a position to modify the receptor by using as templates for RNA synthesis cDNA molecules that we have altered by *in vitro* mutagenesis. Our preliminary results suggest that this will be a powerful tool for studying and eventually modifying the functional receptor. As a start in this work, we have studied the requirements for individual subunits in the receptor oligomer. In a series of experiments we have injected oocytes with RNA molecules encoding various combinations of receptor subunits. Table 1 shows the combinations injected and the fraction of the oocytes giving a measurable response. We obtained functional receptors with all four subunits, with the αβγ combination as reported by Mishina et al. (1985), and with the αβδ combination which has not previously been seen. A more quantitative analysis of the responses is found in Table 2. The wild-type response is the best, but clear responses are seen with the other two combinations. Figure 6B shows that toxin-binding activity can also be detected in these oocytes. We have not seen functional responses with any other combinations of subunits, nor have we been able to create a functional receptor with only the α-subunit. These observations are important because they place constraints upon models of receptor function and because they point out possible difficulties of *in*

FIGURE (6)


A
BC3H-1
αβγδ(−dTC)
αβγδ(+dTC)
B
αβγδ
αβγ
αβδ
125I-α-BUNGAROTOXIN, cpm x 10
0
2
4
6
8
10
12
14
16
18
20
22
24
2 4 6 8 10 12 14 16 18
2 4 6 8 10 12 14 16 18 20
FRACTION NUMBER

JBFIG1.2P, Jim Boulter, MNL, 8/12/86

FIGURE (7)

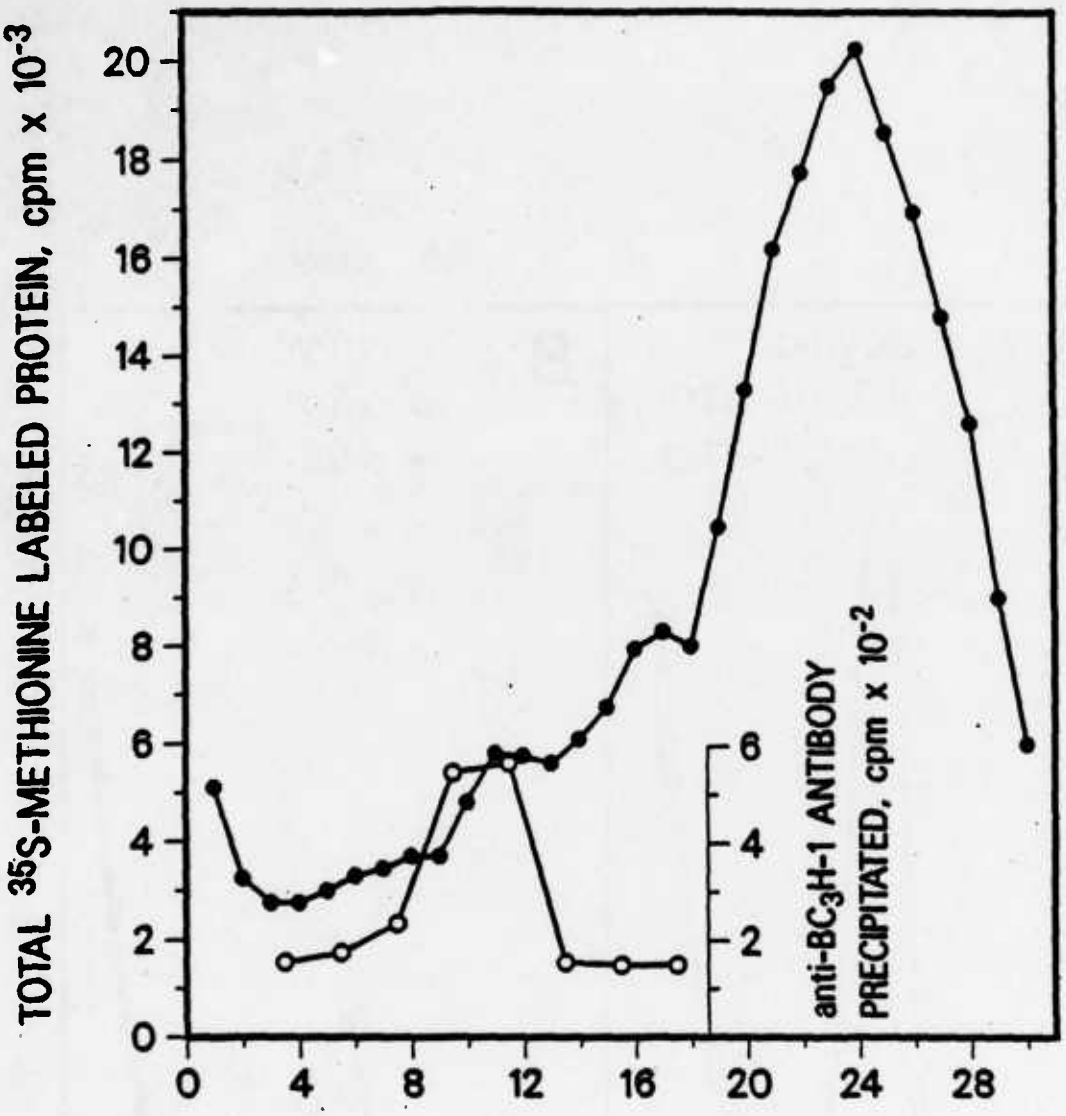


TABLE 1. EFFECT OF SUBUNIT COMPOSITION ON GENERATION OF A FUNCTIONAL RECEPTOR

Subunits	α	αβ	αβγ	αβδ	βγδ	αγδ	αβγδ	none	sham
Response	0/8	1/10	14/16	23/32	0/15	0/22	37/38	0/6	0/15

TABLE 2. SUMMARY OF SUBUNIT COMBINATION RESPONSES TO ACETYLCHOLINE.

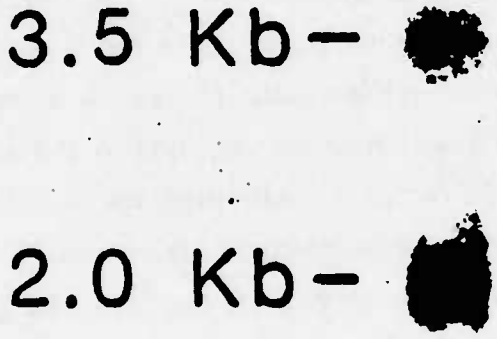
Subunit	Day	Resting Potential	Response	Fraction Responsive	Acetylcholine	Surface Receptor	Total Receptor
αβγδ	1	46.8	32.4	28/29	10^{-6}		
αβγ	1	49.5	9.7	7/9	10^{-6}		
αβδ	1	48.7	2.7	5/13	10^{-4}		
αβγδ	2	57.5	43.8	9/9	10^{-6}	2.4	10.3
αβγ	2	39.1	14.3	5/5	10^{-6}	1.0	5.2
αβδ	2	45.0	12.6	7/12	10^{-4}	1.1	5.2
αβδ	3	65.4	6.5	13/14	10^{-6}		

vitro mutagenesis studies. For example, if we place a mutation in the γ-subunit and find a functional receptor, we will have to prove that the response comes from the altered γ-subunit and not from formation of an αβδ oligomer.

The results outlined above constitute the completion of the first goal of our research program. We have obtained clones encoding all four subunits of a mammalian muscle nicotinic acetylcholine receptor, and the sequences of these clones have been determined. We have also engineered the clones to make them suitable for expression and have shown that they can direct the synthesis of a receptor in the *Xenopus* oocyte. We have also examined the properties of the expressed receptor. In addition, we have created the clones necessary for the creation of mutant receptor subunits and will use this system to test ideas about the structure and function of the mouse muscle nicotinic acetylcholine receptor.

II. NEURONAL NICONITIC ACETYLCHOLINE RECEPTORS

A major goal in obtaining clones coding for the mouse muscle nicotinic receptor was to use these clones to detect, by low stringency DNA/DNA hybridization, sequences coding for possible neuronal nicotinic acetylcholine receptors. We began this program using the PC12 cell line as a source of neural type nicotinic acetylcholine receptors (Greene and Teschler, 1976). On its surface, this cell line has both a neural type nicotinic receptor and an α-bungarotoxin-binding molecule. Both pharmacological (Patrick and Stallcup, 1977b) and immunological (Patrick and Stallcup, 1977a) studies have shown that these are distinct molecular species. The physiological role of the α-bungarotoxin-binding component on these cells is not known. We prepared poly(A)$^+$ RNA from the PC12 cell line and found that a probe prepared from the mouse muscle α-subunit clone hybridizes to two size classes of RNA species, as seen in Figure 8 (Boulter et al., 1986a). The hybrids were not stable at high stringency, suggesting that the bands we detected represented partially but not completely homologous sequences.



PC12 RNA A(+)
PROBE: pMAR α15

851957

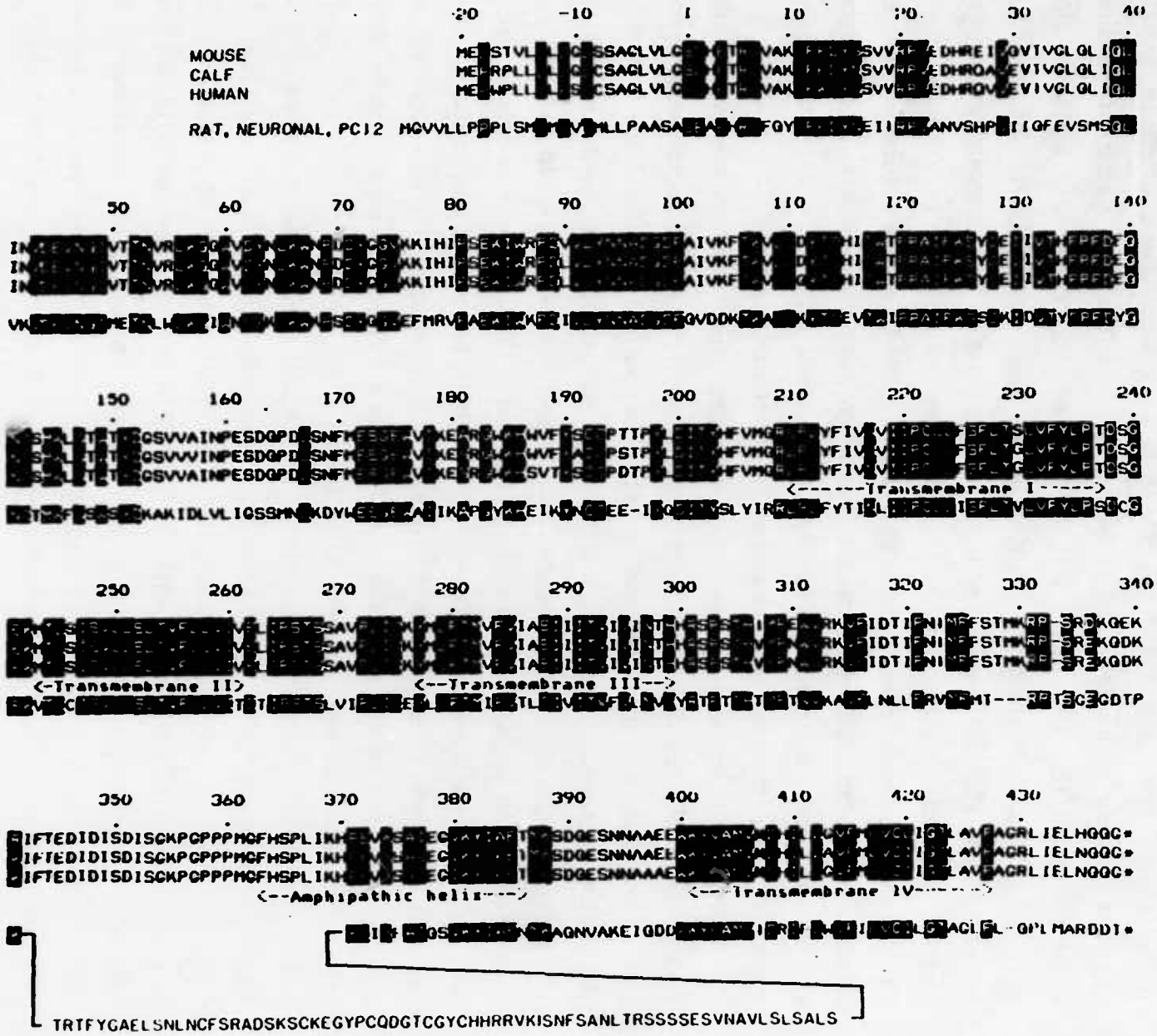
cDNA was prepared from the PC12 poly(A)$^+$ RNA and cloned into the vector λgt10, and the resulting library was screened with a probe corresponding to a conserved region of the mouse α-subunit. This screening, which was done at low stringency, should have detected molecules which differed by as much as 30% in their sequences. Three clones were found and purified. Subsequent restriction site mapping suggested that the clones were similar if not identical and the longest, PCA48, was chosen for sequencing.

Clone PCA48 was sequenced and found to encode the precursor to a protein which we propose, for the following reasons, is an α-subunit of a neuronal nicotinic acetylcholine receptor. The sequence of this protein, aligned with sequences of other α-subunits, is shown in Figure 9. The encoded protein has the four hydrophobic sequences thought to form transmembrane segments in the muscle α-subunit and, in this respect, is structurally similar to the muscle receptor α-subunit. The protein encoded by PCA48 also contains a sequence homologous to the amphipathic helix found in the α-subunits of muscle receptor. Although the sequence homology is not as great as in the hydrophobic membrane-spanning regions, the distribution of charged and uncharged residues about the helix is similar. The glycosylation site found in the proposed extracellular domain of the muscle α-subunit is also found in the PCA48. There is an additional potential glycosylation site in the PCA48 protein at asparagine 24. Finally, the four cysteine residues in the proposed extracellular domain that are conserved in all muscle α-subunits sequenced to date are also found in the PCA48 protein. These observations are all consistent with the idea that the PCA48 protein is a neural nicotinic acetylcholine receptor α-subunit but do not rule out other alternatives.

For example, the protein encoded by PCA48 might be a rat muscle α-subunit. This was ruled out by the observation that a probe prepared from PCA48 does not hybridize at high stringency to RNA prepared from innervated or denervated rat muscle. Furthermore, rat muscle RNA does not protect PCA48 DNA from digestion by endonuclease S1. In contrast, a probe prepared from PCA48 recognizes RNA species in rat brain and in rat adrenal medulla but not in adrenal cortex.

We tried to gain insight into the function of the protein encoded by PCA48 by determining where the gene is expressed. Clone PCA48 was obtained from a library.

Interspecies Comparison of Aligned Amino Acid Sequences
the Alpha Subunit of Muscle and Nerve Acetylcholine Receptor



created from poly(A)$^+$ RNA prepared from the PC12 cell line which has both a nicotinic acetylcholine receptor and an α-bungarotoxin-binding component. Although the protein encoded by PCA48 is homologous to the mouse muscle nicotinic receptor α-subunit, it might in fact represent the α-bungarotoxin-binding component of this cell line. We tried to test this possibility using *in situ* hybridization to determine the distribution in mouse and rat brain of the RNA homologous to PCA48 (Goldman et al., 1986). Sections of rat and mouse brain were probed with radiolabeled single strand RNA derived from the rat neuronal clone PCA48 and the mouse muscle clone BMA407. Figure 10 shows autoradiograms of mouse brain hybridized with a mouse muscle probe (A) or the rat neuronal probe (B), and rat brain hybridized with the rat neuronal probe (C) or control probe consisting of the message sense strand of the rat neuronal clone.

The results in these figures show that probes prepared from the muscle and neuronal clones hybridize to different regions of the brain. The neuronal probe hybridizes most strongly to the medial habenula and to a clear but lesser extent to the ventral tegmental area, substantia nigra pars compacta, anteroventral nucleus of the thalamus, the medial geniculate nucleus and the neocortex. In contrast, the muscle α-subunit probe shows little hybridization to these areas. The dentate gyrus and hippocampus show a positive signal with both the neural and muscle probes but also show hybridization with the control probe, corresponding to a sense strand which should not hybridize to the mRNA. The hybridization seen in these areas might therefore represent nonspecific binding.

The hybridization results show that RNA species homologous to the neuronal clone are found in the brain but do not show that the RNA is identical. The question of identity was addressed by S1 analysis of poly(A)$^+$ RNA isolated from the habenula. This RNA, along with poly(A)$^+$ RNA isolated from the PC12 cell line, was size fractionated on denaturing formaldehyde agarose gels and transferred to Gene Screen Plus. Transcripts hybridizing to the neuronal probe were found in both RNA preparations. Heteroduplexes formed between the neural α-subunit and RNA isolated from either the PC12 cell line or the habenula afforded complete protection of the cDNA clone to S1 nuclease digestion, as seen in Figure 11. This shows that

FIGURE (10)

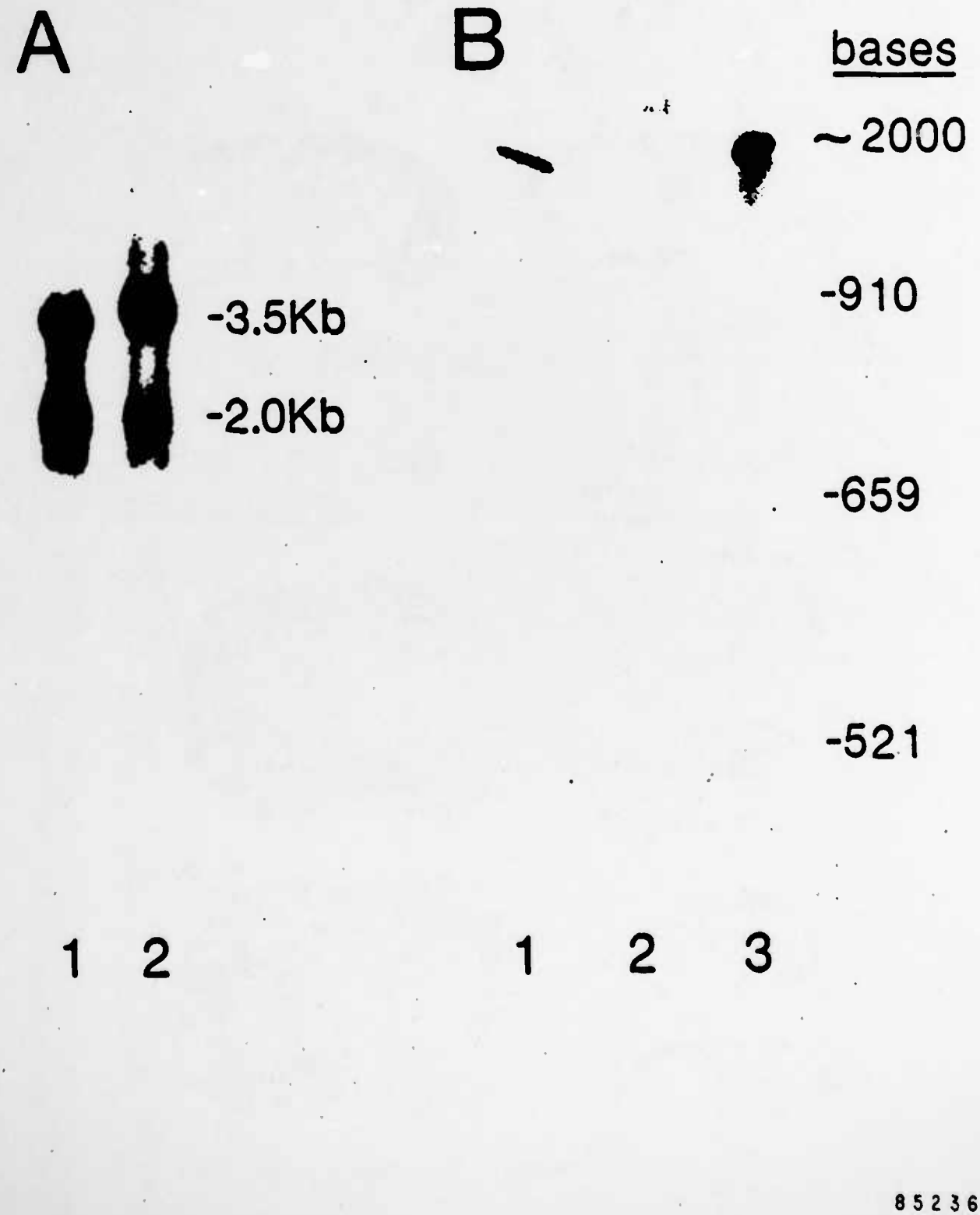


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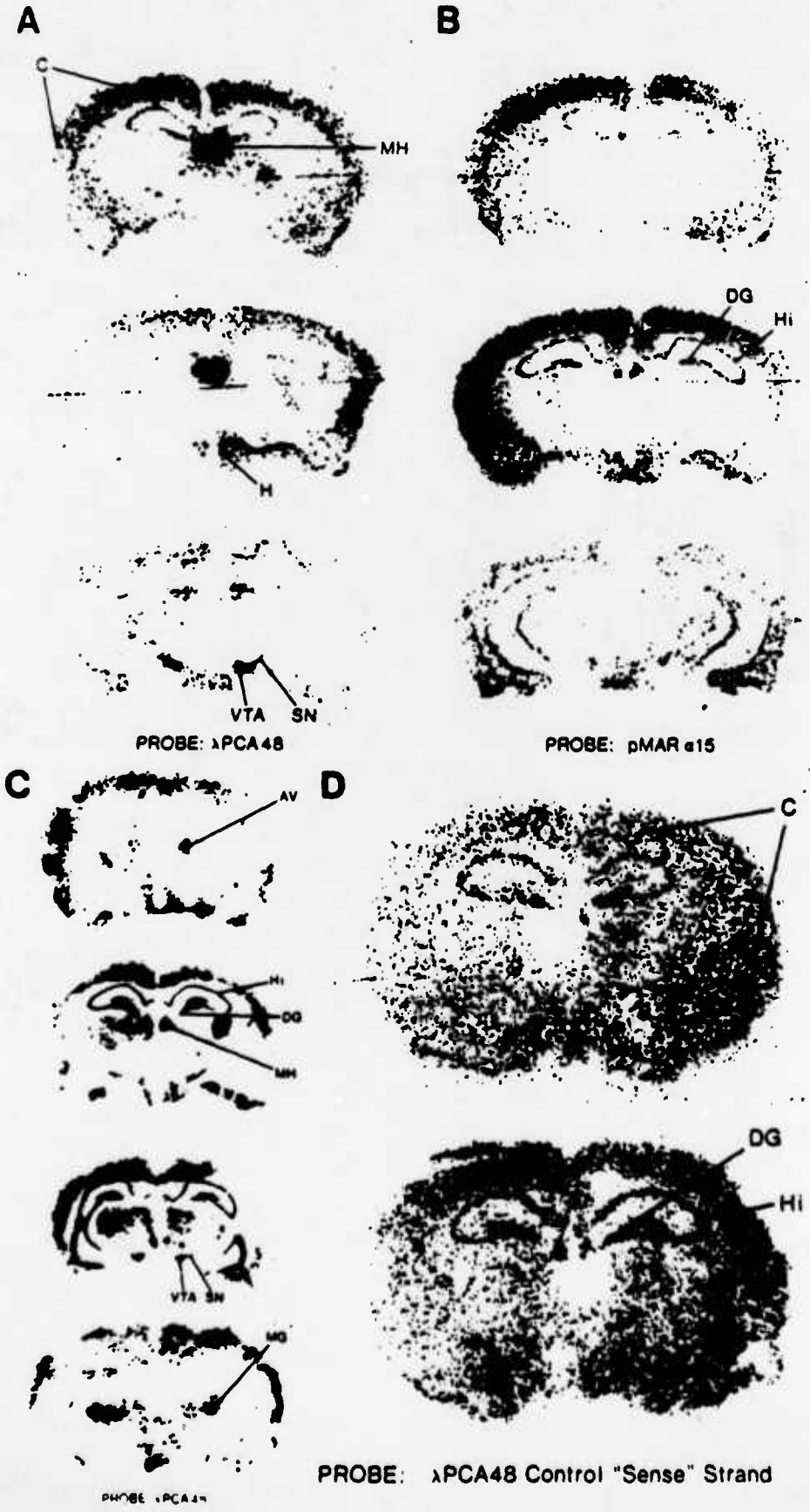
FIGURE (11)



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cells in the medial habenula express the same gene as is expressed in the PC12 cell line and suggests that the hybridization we see to this area of the brain is a consequence of this specific RNA species.

The fact that the medial habenula expresses the gene from which the PCA48 neuronal clone was derived suggests that this clone encodes an α-subunit of a nicotinic acetylcholine receptor rather than the α-bungarotoxin-binding component present on the PC12 cell line. Pharmacological studies show that the medial habenula binds cholinergic ligands but does not bind α-bungarotoxin (Hunt and Schmidt, 1978; Clarke et al., 1985). The argument, however, is slightly more complex because cholinergic neurons in the medial habenula project, via the fasciculous retroflexus, to the interpeduncular nucleus (Herkenham and Nauta, 1979; Gottesfeld and Jacobowitz, 1979) where the amount of α-bungarotoxin binding is controversial (Hunt and Schmidt, 1978; Clarke, 1985). Hence, the hybridizing RNA species seen in the medial habenula could code for the nicotinic receptor in the habenula or for the α-bungarotoxin-binding component thought to be in the interpeduncular nucleus. Antibodies directed against the protein encoded by the PCA48 clone should help distinguish between these possibilities.

The *in situ* hybridizations, Northern blots, and S1 protection experiments suggest that the PCA48 sequence is expressed in the medial habenula and that homologous or identical sequences are expressed in other brain regions. In addition, Southern blots of genomic DNA suggest that there are several different homologous sequences in the genome (Boulter et al., 1986). Since these sorts of analyses can be misleading, the only sure way to identify the hybridizing species and determine their relatedness is by cloning. Therefore, we purified poly(A)$^+$ RNA from specific brain regions, made cDNA libraries, and isolated clones hybridizing to either our muscle or neuronal α-subunit clones. We found hybridizing, partially homologous but nonidentical clones in libraries prepared from the hippocampus, the thalamus, and the cerebellum. These clones define two and possibly four additional α-subunit-like molecules. The role that these molecules play in synaptic transmission in the central nervous system is unknown.

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MATERIALS AND METHODS

RNA Isolation:

RNA was isolated as previously described (Goldman et al., 1985). Briefly, 1-2 grams of tissue were homogenized in buffered guanidine thiocyanate. After clarification, the homogenate was layered over a cushion of CsCl and centrifuged 15 hours at 35,000 rpm in a Beckman SW41 rotor. The RNA pellet was resuspended in water to which guanidine hydrochloride was added and then ethanol precipitated. The RNA precipitate was resuspended in water and ethanol precipitated again. Poly(A)$^+$ RNA was selected by chromatography over an oligo(dT)-cellulose column (Aviv and Leder, 1972).

Construction and Screening of cDNA Libraries:

Two cDNA libraries were constructed using poly(A)$^+$ RNA isolated from the hippocampus or a hypothalamic punch. The method of Gubler and Hoffman (1983) was used to prepare size-fractionated double-stranded cDNA. The cDNA was ligated to phosphorylated Eco R1 linkers and cloned into the Eco R1 site of bacteriophage λgt10 (Huynn et al., 1985). Approximately 5X 10^5 plaques were screened from each library with a radiolabelled cDNA fragment coding for the mouse muscle acetylcholine receptor α-subunit (Boulter et al., 1985), as well as a probe made from the cDNA coding for the α3 gene product (Boulter et al., 1986).

DNA Sequence Determination:

DNA sequencing was performed using the dideoxynucleotide chain termination method of Sanger et al. (1977). cDNAs were subcloned into M13 bacteriophage vectors mp18 and mp19. Deletions were generated by the method of Dale et al. (1985).

RNA Blots:

RNA was denatured in formaldehyde at 65°C and electrophoresed in 2.2M formaldehyde, 1.4% agarose gels. The RNA was then transferred to a Gene Screen Plus membrane. Prehybridization and hybridization conditions were 5X SSPE

(0.75M NaCl, 57mM Na_2HPO_4, 5mM EDTA, pH 7.4), 1% SDS, 10% dextran sulfate, and 50% formamide at 42°C. After hybridization the blot was washed in 0.2X SSPE, 1% SDS at 65°C and was exposed to X-ray film with an intensify ing screen at -70°C.

S1 Nuclease Analysis:

Nuclease S1 digestions of heteroduplexes formed between poly(A)$^+$ RNA and M13 subclones of the α4 cDNA clone were carried out as described (Goldman et al., 1985). The 3' 596 nucleotides of the α4 cDNA were subcloned into M13mp18 and the single-strand viral DNA was used to form heteroduplexes. Those hybrids surviving S1 nuclease digestion were analyzed by electrophoresis through a 3% polyacrylamide-8M urea gel, electroblotted to Gene Screen Plus and detected by hybridization to nick-translated radiolabelled α4 cDNA.

In Situ Hybridization:

In situ hybridization was performed as previously described (Cox et al., 1984; Goldman et al., 1986). Briefly, brain sections mounted on polylysine coated slides were treated with proteinase K, acetylated with acetic anhydride and dehydrated prior to hybridization. Sections were hybridized with single strand radiolabelled RNA probes prepared from an SP6 vector containing a cDNA insert encoding either the α3 or α4 gene product. Hybridization was performed at 42°C for 14-18 hours. Post-hybridization treatments included RNase A digestion and a final wash in 0.1X SSPE at 65°C. Slides were dehydrated and exposed to X-ray film at room temperature for 3-20 days.

Sequence Alignment and Homology Calculations:

Protein sequences were aligned using an INTELLIGENETICS software IFIND program that utilizes an algorithm developed by Wilbur and Lipman (1983). Parameters were set to default values. Alignments were adjusted by visual inspection. Homology percentages were calculated by dividing the number of identical residues by the number of residues in the shorter of the two sequences being compared.

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